SHORT COMMUNICATION

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In vitro assessment of the cytotoxic effects of novel RGD analogues

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Abstract

The RGD sequence is present in many extracellular matrix proteins and intracellular proteins, including caspases. Synthetic RGD peptides may affect adhesion, migration and tumour metastasis, or directly induce apoptosis. Several RGD peptides were synthesized, and their anti-adhesive and cytotoxic properties were analyzed *in vitro*. Here we present the cytotoxic activities of RGD, R(NO_2)GD, CavGD and RGD-OMe on non-tumour 3T3 cells and tumour cell lines HepG2 and MCF-7. The cell growth inhibitory effects of RGD-OMe are significantly higher than those of RGD on the cell lines used. Evidently the modification in the carboxylic group of RGD with simple esterification increases the cell growth inhibitory effects of the parent compound.

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Introduction

Among the peptide family, short peptides are very appealing for drug discovery and development because of their cost-effectiveness, possibility of oral administration, and simplicity to perform molecular structural and quantitative structure-activity studies [1]. Small peptides are under development as possible anti-tumour agents as well. Examples of these are peptides with RGD (L-argininylglycyl-L-aspartic acid) or NGR (L-aspartylglycine-L-arginine) motifs presented in fibronectin, laminin, vitronectin, fibrinogen and collagens [2,3]. The discovery of the minimal peptide sequence RGD, which plays a prominent role in cell adhesion via integrin interaction, has led to a large increase in biomedical and biomaterials research on this motif. RGD peptides are commonly known as antimetastatic agents and are able to decrease the number of spontaneous and experimental metastases in *in vivo* models [4-7]. Moreover, descriptions exist of some of their *in vitro* activities, like inhibition of tumour cell adhesion to extracellular matrix proteins and the inhibition of migration in a three dimensional gel [6, 8, 9].

Various RGD-containing peptides have been increasingly developed for adapting to versatile applications including tumor imaging and therapy, drug delivery vector, targeted gene transfer, and biomaterial or tissue engineering. Significant progress has been

made in the discovery and development of integrin $\alpha_v\alpha_3$-specific linear and cyclic RGD peptide analogs such as cilengitide and c(RGDfK) for cancer therapy, as well as targeted delivery of cancer imaging and therapeutic agents. Examples of such radio-imaging agents include [99mTc]apticide, which can be used in imaging deep vein thrombosis [10]. Several modifications of RGD peptides, including polymerisation, coupling with carriers and substitution by peptidomimetics, were performed to enhance the anti-tumour properties and lengthen the degradation time *in vivo* [11-14]. RGD peptides conjugated with different cytostatic agents are likely to exhibit an antitumour and antiangiogenic synergetic effect. During the last few years, a number of RGD-cytotoxic drugs were developed and showed promising activities *in vitro* and *in vivo* [15,16].

Despite the few RGD analogues being approved for clinical use, development of orally active RGD peptidomimetics have been significantly hindered because of low bioavailabilities. This is largely due to the metabolic lability of this class of compounds in the presence of proteases and peptidases and because of their high polarity and charge. Wang et al. [17] published a detailed review of peptidomimetic analogues of RGD and the strategies used to enhance their bioavailability.

In this context, drug design based on the RGD structure may provide opportunity for targeted drug deliveries, high resolution imaging of cancerous tissues and organs, as well as new chemotherapeutic treatments for cancer.

Following our long-term program for the design of biologically active peptides based on the non-protein amino acids, we synthesized several short RGD-mimetics containing the sequence **Xaa-GD**, where **Xaa** is Arg or Arg-mimetic (Figure 1) with a view to improve its cytotoxic activity [18].

The purpose of this study, therefore was to investigate the cytotoxic activities of RGD and its newly synthesized mimetics (2-4) on non-tumour 3T3 cells and tumour cell lines HepG2 and MCF-7.

Results

The RGD analogues were synthesized following a procedure previously reported [18]. For the synthesis of peptides **1-3** standard Fmoc-SPPS was applied using HBTU as a coupling reagent. Peptide deprotection and cleavage from the resin were carried out in a mixture of TFA and scavengers. The peptide **4** was prepared by classical solution procedure. We were able to obtain the RGD mimetics with satisfactory purities (typically >95%), as assessed by analytical HPLC (Figure 2). Furthermore the stability of the peptides (**2-4**) has been proven during 24 h period. The peptide analogues were quite stable in aqueous solution. For example, the decomposition of RGD-OMe after 24 h incubation in culture medium at 37°C was less than 2%, as assessed by UV-spectroscopy and analytical HPLC.

The results of the cytotoxicity of RGD and RGD-OMe on non-tumour 3T3 cells and tumour cell lines HepG2 and MCF-7 are shown in Figure 3. The cells were exposed for 24 hours to different concentrations (ranging from 2 to 0.015 mM) of the compounds. As seen in Figure 3. treatment of the cell lines resulted in a dose-dependent reduction of the number of the viable cells. The RGD analogue RGD-OMe exhibited higher cell growth inhibitory effects on the three cell lines compared to its parent compound. Statistically reliable results for the cytotoxic action of RGD-OMe on HepG2 and MCF-7 cells were achieved for the most of concentrations used.

The cytotoxic activity of the synthesized peptides

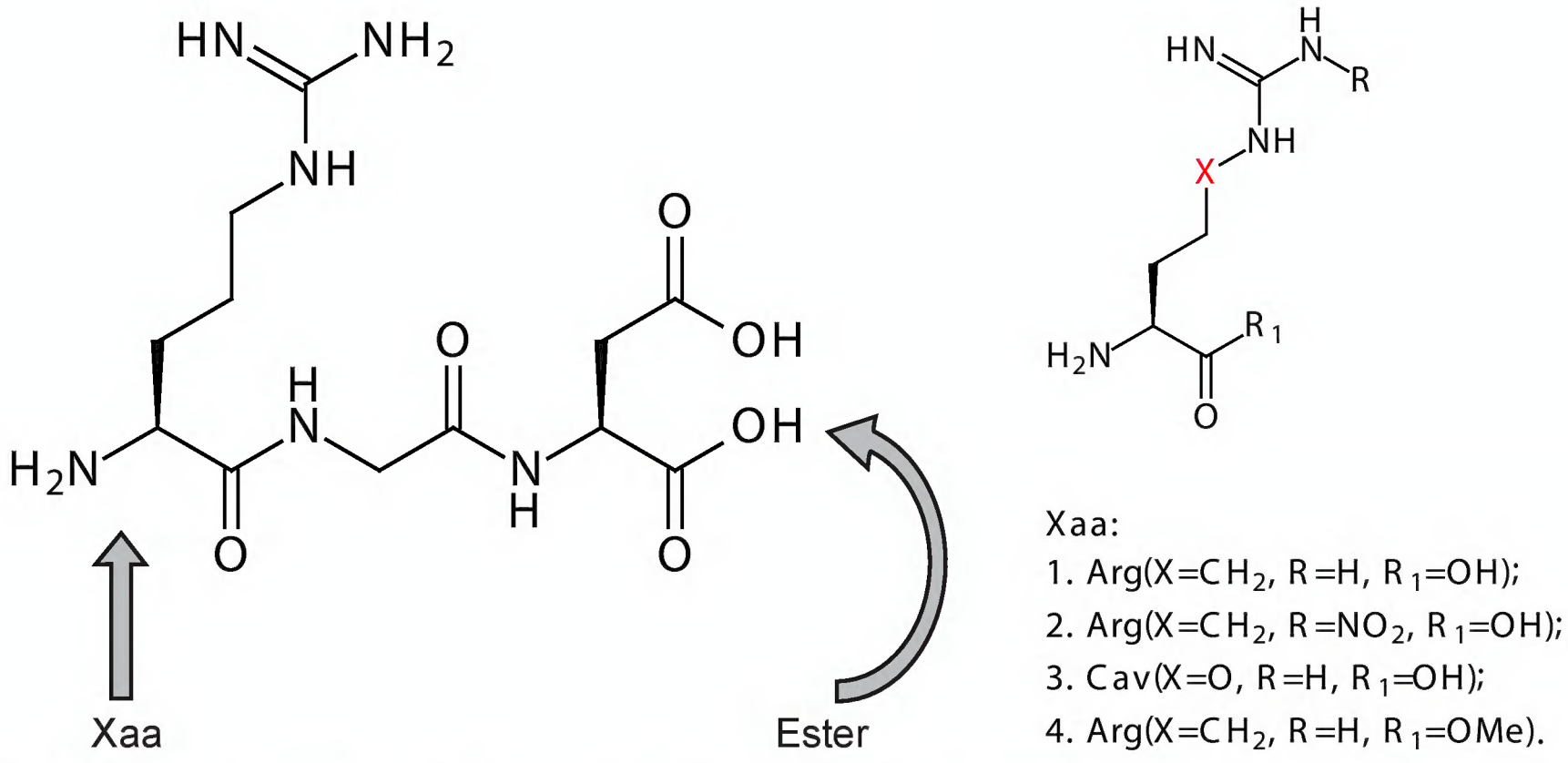

Figure 1. Structure of RGD and analogues: Arg-Gly-Asp (RGD, **1**); Arg(NO2)-Gly-Asp (R(NO_2)GD, **2**); Cav-Gly-Asp (CavGD, **3**); Arg-Gly-Asp-methyl ester (RGD-OMe **4**).

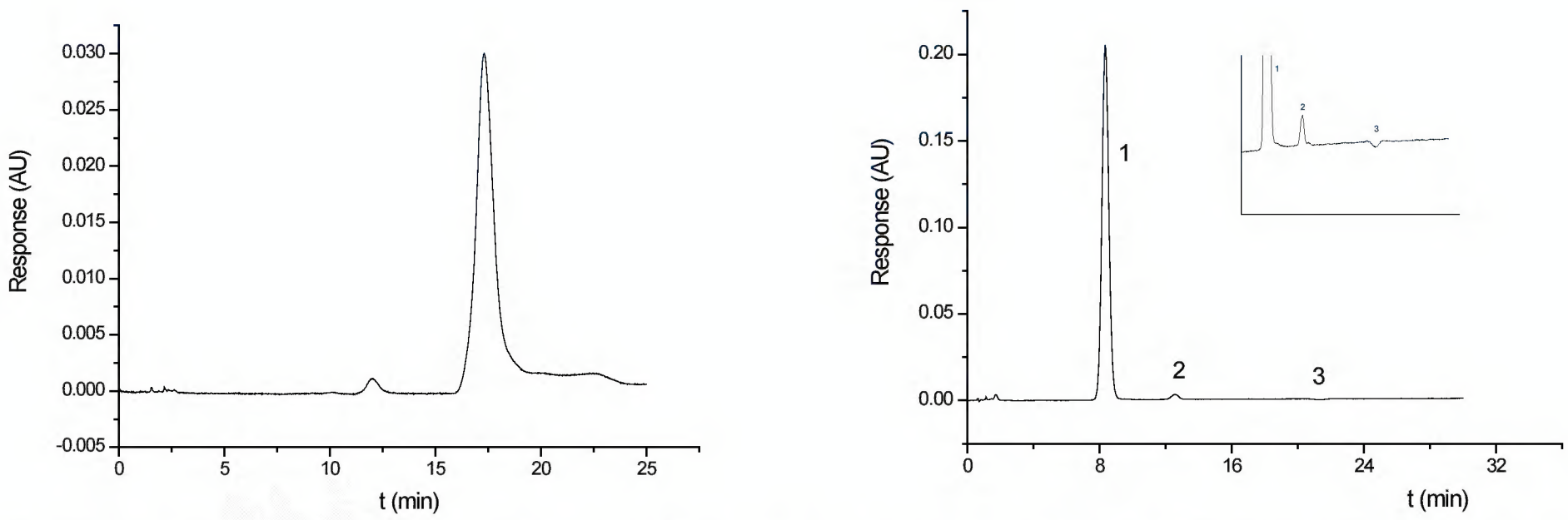

Figure 2. HPLC-chromatograms of peptides **3** and **4**. Chromatographic conditions: column: Atlantis^{TM}dC$_{18}$, 4.6×150 mm, particle size 5µm, mobile phase: acetonitrile/deionised water 40/60 (v/v), column and sample temperature: 25°C, flow rate: 1ml/min, UV detection - 206 nm.

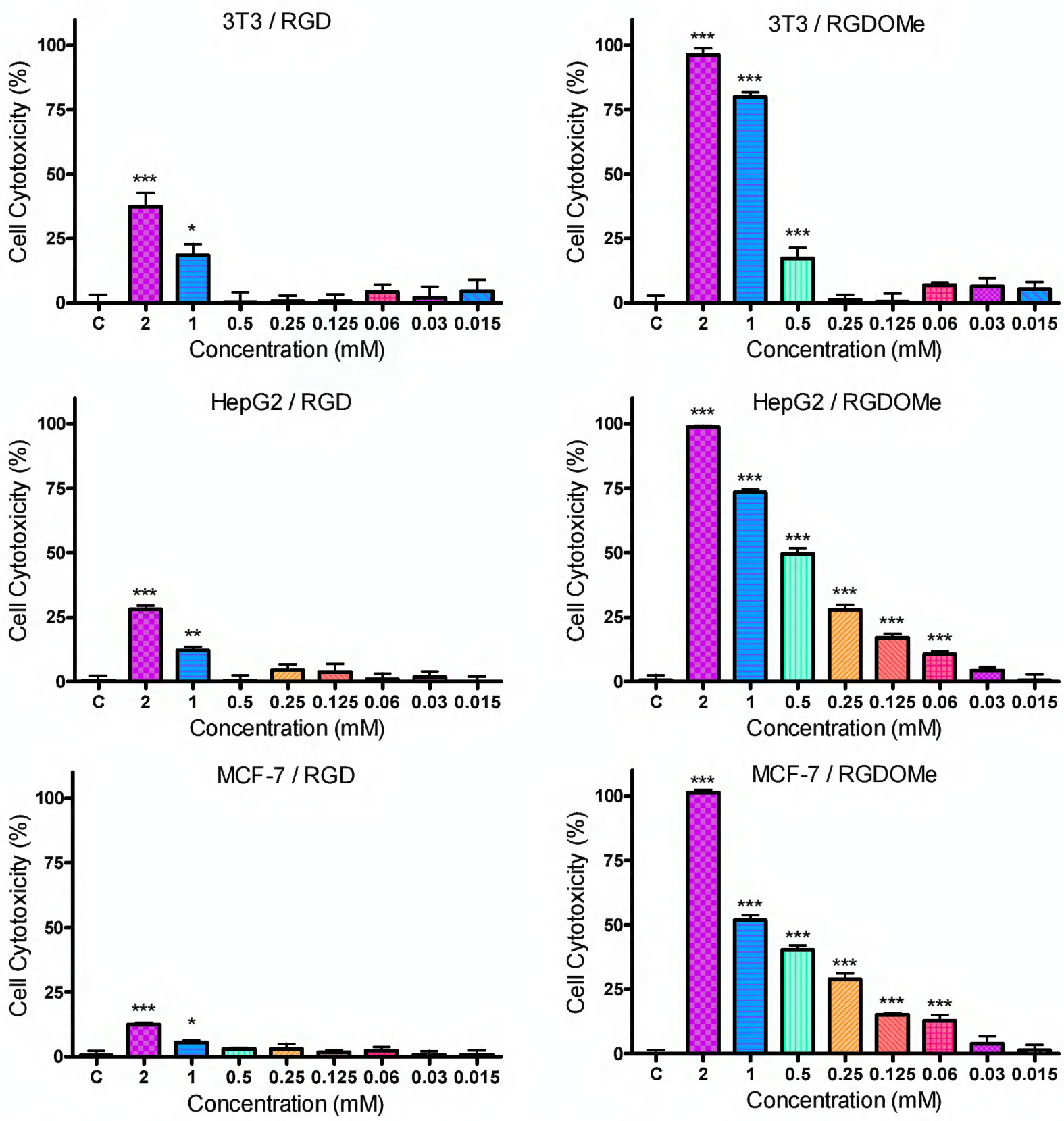

Figure 3. Effect of RGD and RGD-OMe on growth of 3T3, HepG2 and MCF-7 cells after 24 h of treatment. Cell cytotoxicity determined by MTT assay is expressed as per cent of dead cells and presented as mean ± SD (n=6), ***P<0.001, **P< 0.01, *P<0.05, ANOVA-test, versus the control group. C – control.

(**1-4**) is demonstrated by calculating the half maximal inhibitory concentrations (IC_{50} values), shown in Table 1. IC50 values of **1-3**, in 3T3, HepG2 and MCF-7 are >2 mM (Table 1). IC50 values of RGD-OMe in 3T3, HepG2 and MCF-7 are 0.758, 0.524 and 0.927 respectively. Evidently HepG2 is the most sensitive of the three cell lines to the cytotoxic effects of the RGD-OMe.

Table 1. Comparative cytotoxic activity of RGD and its analogues R(NO_2)GD, CavGD and RGD-OMe in 3T3, HepG2 and MCF-7 cells after 24 h treatment (MTT-dye reduction assay).

Peptides	Mean IC_{50} values (mM)*		
	3T3	HepG2	MCF-7
RGD	>2	>2	>2
R(NO_2)GD	>2	>2	>2
CavGD	>2	>2	>2
RGD-OMe	0.758±0.0504	0.524±0.0766	0.927±0.1066

HepG2 cells, treated with RGD and RGD-OMe were investigated for damages in their genomic DNA by neutral variant of Comet assay. The obtained by us Comet assay data confirmed that the RGD and RGD-OMe revealed certain DNA damaging activity, i.e. genotoxicity, which was increasing with the modifications of the compound (results not published). In an attempt to look deeper in the details of the observed genotoxicity we searched for consequence of the compounds on the cell cycle. The FACS analysis, however, has not proposed evidence of distinctive influence of the studied compounds on the stages of cell cycle. This result show that even strongly pronounced the genotoxic activity of the compounds is not connected with certain check point of the cell cycle (results not published).

Discussion

Peptides containing unusual amino acids are used in a multitude of applications including structural activity studies, diagnostics, and new drug discovery, improvements in bioavailability, molecular markers, and biologically active pharmaceuticals.

We decided to carry out various structural modifications on N- and C-terminal of the RGD molecule [18]. We chose for example, unnatural amino acid L-canavanine (**Cav**), as a promising candidate for the synthesis of RGD analogues, instead of the basic residue Arg.

As shown by several authors, **Cav** a structural analogue of Arg, possesses growth retardation activity toward tumor cells in culture and experimental tumors *in vivo* [19-21]. We supposed that modification with the structural analogue and antimetabolite of Arg, will improve the antitumor activity of the parent molecule.

The cytotoxic activities of peptides **2** and **3** on 3T3 and HepG2 cells were examined (results not shown) but only the highest concentration used (2 mM) revealed cell growth inhibitory effect. Next, we decided to perform an experiment with C-terminal modified RGD. For this purpose we synthesized RGD-OMe **4**.

The results showed that the cell growth inhibitory effects of **4** are significantly higher than those of RGD on the three cell lines. Evidently, the modification in the carboxylic group of RGD with simple esterification increases the cell growth inhibitory effects of the parent compound.

The effect of RGD-OMe is higher in the case of the tumor cells HepG2 in comparison with non-tumour cells 3T3. This observation suggests that the modification in the carbocylic group is significant for the antitumour action of RGD.

According to our recent investigations, as well as our further work, which is in progress, these new members of RGD are easily transformed into derivatives with improved pharmacological properties, or they can be used as building blocks for combinatorial peptide libraries.

Materials and Methods

Synthesis

The peptides **1** and **2** were synthesized manually by the solid phase method using Fmoc chemistry on (2-Chloro) chlorotrityl resin (Iris Biotech GmbH, Germany) using Fmoc/tBu methodology. Fmoc groups were removed using 20% piperidine in DMF. Coupling reactions were performed using Fmoc-amino acid/HBTU/HOBt/DIEA/ resin in molar ratio of 3/3/3/9/1. The coupling and deprotection reactions were checked by the Kaiser test. Peptide removal from the resin and the removal of side chain protection groups were performed using 95% TFA with 0.3% TES treatment. The synthesis of RGD-OMe was prepared by classical solution procedure using mixed anhydride coupling reactions. The final products were purified by gel filtration on a Sephadex G-10 column. The peptide purity was monitored on a RP-HPLC column Atlantis^{TM}dC_{18}, 4.6×150 mm, particle size 5µm, mobile phase: acetonitrile/deionised water 40/60 (v/v), at 25°C, flow rate: 1ml/min, and UV detection - 206 nm. The correct molecular masses were confirmed by ESI-MS. For R(NO_2)GD, **GF** - $C_{12}H_{21}O_7N_7$, $[MH]^+$ calculated -375.34, $[MH]^+$ observed -375.36; CavGD, **GF** - $C_{11}H_{21}O_6N_6$, $[MH]^+$ calculated -333.32, $[MH]^+$ observed -333.19; RGD-OMe, **GF** - $C_{13}H_{24}O_5N_6$, $[MH]^+$ calculated -344.37, $[MH]^+$ observed -344.59.

Cell cultures

The 3T3 (standard mouse embryonic fibroblast cell line), MCF-7 (human breast cancer cell line) and HepG2

(human liver hepatocellular carcinoma cell line) cells were cultured in Dulbecco Modified Eagle's medium (DMEM) (Gibco, Austria) supplemented with 10% fetal bovine serum (Gibco, Austria), 100 U/ml penicillin (Lonza, Belgium) and 0.1 mg/ml streptomycin (Lonza, Belgium) under a humidified 5% CO_2 atmosphere at 37°C. Plastic flasks supplied by Greiner, Germany, were used to grow the cells. Cells were trypsinized using Trypsin-EDTA (FlowLab, Australia) when they reached approximately 80% confluence. For experiments the cells in exponential phase of growth after treatment with Trypsin-EDTA were seeded into 96-well plates (Greiner, Germany) in a concentration $2x10^4$ cells/well. 24 hours incubation post seeding (under a humidified 5% CO_2 atmosphere at 37°C) allowed the cells to attach to the wells.

Cytotoxicity assay

The cultivated cells were treated with RGD and its analogues (**2-4**) in a wide concentration range (2 - 0.015 mM). Untreated cells were used as controls. Empty wells were blank controls. Cytotoxicity was measured by colorimetric assay based on tetrasolium salt MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma Chemical Co.). The MTT assay is based on the protocol first described by Mossman (1983). In this assay, living cells reduce the yellow MTT to insoluble purple formazan crystals. The peptides were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in samples did not affect the viability of the cells. The assay was performed 24 hours after treatment with the amino acid analogues. For this purpose, MTT solution was prepared at 5 mg/ml in PBS and was filtered through a 0.2 µm filter. Then 1 ml of MTT solution was added to 15 ml DMEM and 100 µl of this solution were added into each well, including the cell free blank wells. Then the plates were further incubated for 3 hours to allow MTT to be metabolized and the supernatant was removed. 100 µl/well DMSO/etanol (1/1) was added. The plates were placed in a microtitre-plate shaker for 10 min at room temperature to thoroughly mix the purple formazan into the solvent. ELIZA plate reader (TECAN, Sunrise TM, Grodig/Sazburg, Austria) was used for reading the results. Optical density (OD) was determined at a wavelength of 540 nm and a reference wavelength of 620 nm. Cell cytotoxicity determined by MTT assay was expressed as per cent of dead cells:

% cytotoxicity = (1 – (OD sample – OD blank control)/ (OD control – OD blank control)) x 100

PrizmaPlot.4 (ANOVA-test) was used for statistical analysis.

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